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Comparison of Two Nested PCR, Cell Culture, and Antigen Detection for the Diagnosis of Upper Respiratory Tract Infections due to Influenza Viruses

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Influenza surveillance requires sensitive and rapid diagnostic methods. Different diagnostic procedures have been evaluated on a selected set of nasal swabs sample collected from patients presenting with acute respiratory infection. One hundred fifty-four samples collected during the peak of the influenza epidemic recorded during winter of 1997–1998 in the south of France were processed for influenza detection using antigen detection (ELISA-immunocapture assay), two different nested RT–PCR assays (targeting M and HA genes), and cell culture. Among 154 samples, 93 (60.4%) were positive for influenza detection. Forty specimens (26%) were positive by ELISA, 77 (50%) by culture, 88 (57.1%) using the multiplex HA-PCR and 76 (49.4%) using the M-PCR. Multiplex HA-PCR was thus the most sensitive test. The PCR assay offers an alternative to culture for influenza detection. Nevertheless, culture is efficient for influenza diagnosis and is the only technique that allows the reference centres to collect viral strains and characterise fully new variants. ***J. Med. Virol. 59:215–220, 1999.***

KEY WORDS: influenza; diagnostic procedure; evaluation; polymerase chain reaction; tissue cell culture; ELISA

INTRODUCTION

Influenza viruses, major agents of respiratory diseases, are responsible for epidemics resulting in excess mortality and morbidity every year. Optimal influenza surveillance requires a system of networks that combine epidemiological and virological data [Lina et al., 1996; Ellis et al., 1997; Zambon, 1998]. This combined surveillance, performed under the auspices and guidelines of the WHO, allows the National Reference Centres to monitor influenza epidemics and characterise fully influenza strains in various parts of the world [Anonymous, 1997; Zambon, 1998]. Most of these reference laboratories process nasal swabs or nasal washes collected from patients presenting with signs and symptoms of acute respiratory infection. In addition to rapid antigenic and/or RNA detection, reference centres also cultivate influenza viruses to detect and report the emergence of any relevant variant [Aymard, 1994].

Among the various sensitive and efficient procedures available for rapid influenza detection, RNA detection by PCR has been evaluated as a reliable diagnostic method, comparing favourably with "reference" techniques such as viral culture or antigenic detection using an immunofluorescence assay or an ELISA [Claas et al., 1993; Dominguez et al., 1993; Cherian et al., 1994; Atmar et al., 1996; Ellis et al., 1997].

In our laboratory, nasal swab samples collected by practitioners of the GROG surveillance network are processed for antigenic influenza detection and viral culture [Lina et al., 1996], the latter using an improved method (incorporating a low speed centrifugation step) on Madin Darby Canine Kidney cells (MDCK) as described previously [Chomel et al., 1991]. This procedure has been used to monitor influenza epidemics since 1987. This diagnostic procedure was selected because of the high number of samples processed every year. During the 6 months surveillance, an average of 1,200 swabs are received and examined for detection and culture of influenza viruses as described above. Moreover, such a screening procedure has the

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added advantage that, in combination with the detection of influenza viruses, the presence of other respiratory pathogens is also examined systematically for each sample (respiratory syncytial virus, adenovirus, parainfluenza virus, rhinovirus, enterovirus and *Mycoplasma pneumoniae*) [Lina et al., 1996]. Hence culture is a versatile technique that allows the detection of various respiratory viruses.

Because of the reverse transcription step prior to PCR and the frequent need for a nested procedure, the specific detection of RNA viruses by PCR is expensive compared to conventional techniques such as ELISA or immunofluorescence. On the other hand, PCR is a rapid diagnostic method of very high sensitivity.

In each laboratory, the choice of the technique used for influenza diagnosis is frequently a compromise between the equipment required to undertake the test, the costs of reagents, the number of technicians required to carry out this test, the sensitivity of the test, and the main priorities of the laboratory (need for a rapid diagnosis, need for a full characterisation of isolates).

This study evaluated the respective sensitivity of two nested PCR techniques, an antigen detection assay and culture for the detection of influenza viruses on a set of nasal swab samples collected during the influenza peak of winter 1998. The results highlight the need for a global approach to influenza detection/culture that would encompass both WHO requirements for influenza surveillance and laboratory management for rapid diagnosis of influenza.

MATERIALS AND METHODS

Patients and Specimens

During the 1997–1998 influenza surveillance, 1,291 nasal swabs samples were collected from outpatients presenting with acute respiratory infection (ARI) and sent to the laboratory by mail. Samples were collected using Virocult swabs in order to ensure viability of the viruses [Jensen and Johnson, 1994]. Specimens were taken by 150 general practitioners and 28 paediatricians from the 1st of October 1997 until the 20th of May 1998 in the south of France. This surveillance allowed the National Reference Centre to identify and monitor the influenza epidemic that was recorded in the south of France between week N 5 and week N 15 of the surveillance (Fig. 1). During the early peak of the epidemic (weeks 7, 8, and 9 of the surveillance), the National Reference Centre received 154 swab samples. The comparison of the four techniques was carried out on this fraction of swab samples.

Influenza Virus A and B Detection and Culture

Upon arrival, the swabs were immediately processed as described previously [Lina et al., 1996]. Briefly, they were removed from the transport tube, their contents expressed and broken up in a sterile glass tube containing 2.5 ml of Eagle's Minimum Elementary Medium (Biowhittaker, Verviers, Belgium), and the transport medium was added. The tube was agitated vigorously using a Vortex mixer and then 0.3 ml of a solution containing 200,000 Units of Penicillin G per ml (Biowhittaker, Belgium) and 200 mg of Streptomycin per ml (Biowhittaker, Belgium) was added. Fractions of this suspension was used immediately for viral culture and antigenic detection (0.3 and 0.1 ml, respectively), the remaining volume was frozen at −20°C prior to PCR analysis.

The immunocapture ELISA to detect influenza A or B antigens was conducted on 0.1 ml of the suspension as described previously [Chomel et al., 1991]. In addition, 0.3 ml of the suspension was inoculated onto each of the three cell lines (MDCK, MRC5 and Hep2) used for viral culture [Chomel et al., 1991]. As described previously [Lina et al., 1996], specimens have also been processed for detection or culture of respiratory syncytial virus (ELISA and culture), parainfluenza virus (ELISA and culture), adenovirus (culture), rhinovirus (culture), enterovirus (culture), and *Mycoplasma pneumoniae* (PCR).

Influenza Detection by RT-PCR

Two nested RT-PCR assays were carried out on each sample suspension previously frozen and stored at −20°C (see above). PCR 1 is a multiplex RT-PCR procedure described by Zhang and Evans [1991], designed to detect specifically the haemagglutinin genes of influenza A H1N1, A H3N2, and B viruses within the same reaction tube. PCR2 is derived from the procedure described by Cherian et al. [1994], used to detect the M gene of influenza A viruses only. These two nested RT-PCR were chosen because of their ability to differentiate influenza subtypes (PCR1) and their putative high sensitivity (PCR2).

The RNA extraction and reverse transcription procedures were identical for both PCR assays. Briefly, RNA extraction was carried out on 0.1 ml of the thawed sample suspension. This volume was mixed with 500 μl of lysis buffer containing 5.75 M guanidium thiocyanate, 100 mM beta-mercaptoethanol, and 35 mg/L glycogen (SIGMA, Saint Quentin-Fallavier, France), and was incubated for 5 minutes at room temperature. Ice-cold isopropanol (600 μl) was then added, the mixture was agitated vigorously and the tube was centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed and the pellet washed twice with 1 ml of 70% ice-cold Ethanol. The pellet was dried using the Speed-Vac and resuspended in 20 μl of di-ethyl pyrocarbonate-treated water. To avoid RNA degradation due to RNases, 40U (1 μl) of RNAsine (Promega, Charbonnières, France) was added. Five microliters of the extracted suspension was used subsequently for reverse transcription. RT was performed with AMV-RT (Promega, France) using random hexamers (pd(N)6, Boehringer Mannheim, Meylan, France) at 37°C for 1 hour.

PCR procedures were carried out according to Ellis et al. [1997] (PCR1) and Cherian et al. [1994] (PCR2). Nine microliters of each PCR reaction was mixed with 1 μl of loading buffer and loaded subsequently in a 2%

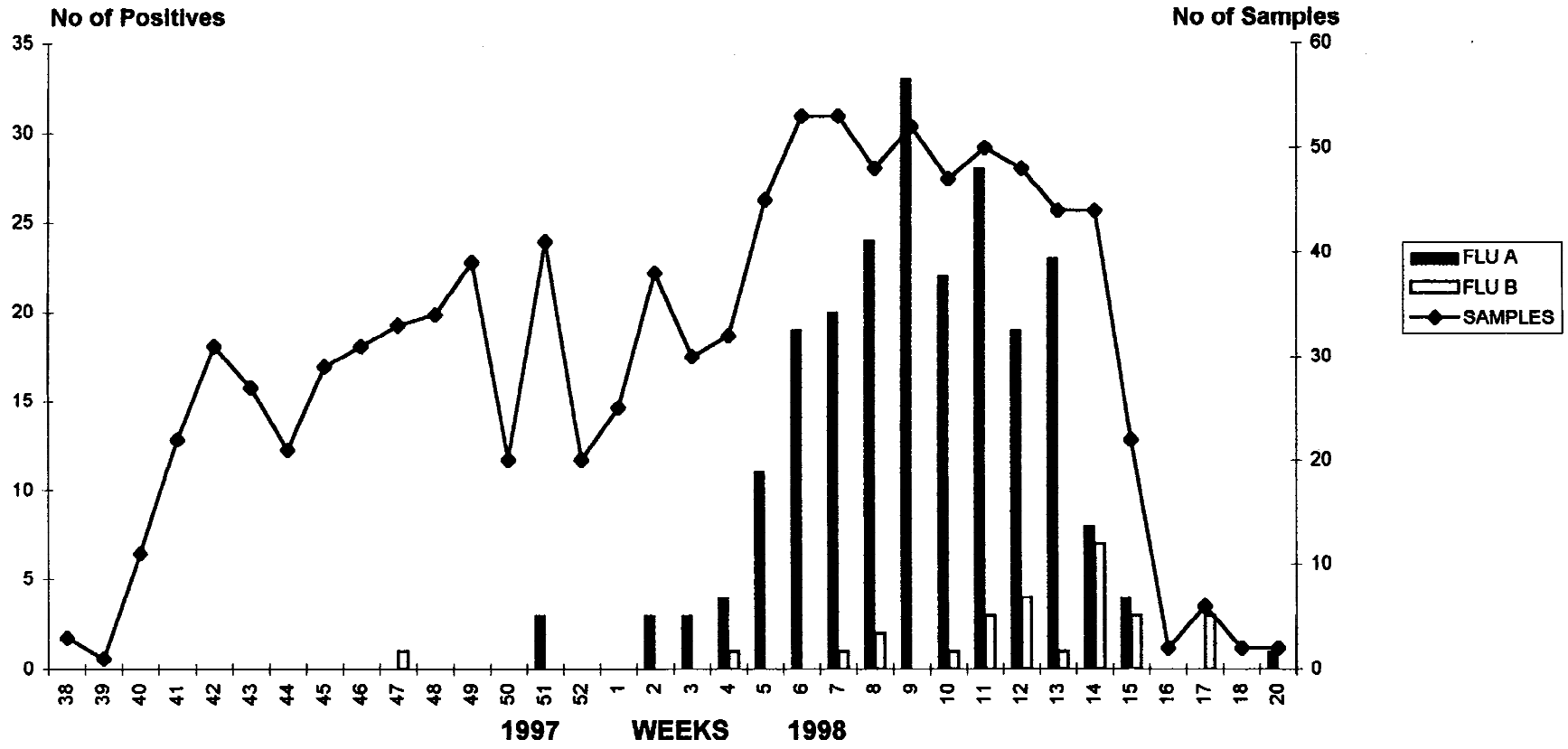

Fig. 1. Result of the influenza surveillance during winter 1997–1998. The surveillance was carried out using influenza detection by IC-ELISA and cell culture. No, number; Flu A, influenza A; Flu B, influenza B.

TABLE I. Comparison of Influenza Detection or Culture on Nasal Swab Specimens Using Four Different Assays

Influenza type	IC-ELISA	Culture	IC-ELISA + Culture	PCR1	PCR2	At least one positive test
A H1N1	0	2	2	2	2	2
A H3N2	38	73	77	84*	74	88*
B	2	2	3	4*	/	5*
Total (%) of positive specimens	40 (26%)	77 (50,6%)	82 (53,6%)	88 (57,1%)	76 (50%)	93 (60,4%)

/, not performed; *, including two A H3N2 and B mixed infections.

agarose gel. Gels were run for 20 minutes at 100 V in 1× TBE buffer (Tris Borate EDTA) and subsequently stained by ethidium bromide and photographed under UV light with a polaroid camera. PCR assays were carried out using a coded numbering system. Two negative controls (water) and three positive controls (strains A H1N1 Beijing 262/95, A H3N2 Sydney 5/97, and B Harbin 7/94) were included systematically in each PCR run. All discrepancies observed between the results obtained with different techniques were re-analysed retrospectively for confirmation of the discrepancy.

Evaluation of the Sensitivity of the Assays

The sensitivity of each test (IC-ELISA, culture, PCR1, and PCR2) was estimated by analysis of 10-fold dilutions of calibrated viral suspensions of influenza A H1N1 Beijing 262/95 ($10^{7.5}$ $TCID_{50}$/ml), A H3N2 Sydney 5/97 ($10^{7.7}$ $TCID_{50}$/ml), and B Harbin 7/94 ($10^{8.5}$ $TCID_{50}$/ml). This evaluation of the sensitivity was carried out twice, and all tests were carried out on the same vial of viral suspension. The viruses were cell adapted strains.

Statistical Analysis

Statistical analysis was carried out using a chi squared test.

RESULTS

Among the 154 samples tested by the four techniques, influenza virus was detected in 93 cases (60.4%) including 2 influenza A H1N1, 88 influenza A H3N2 and 5 influenza B (Table I). In two cases, where PCR1 detected a A H3N2 and B mixed infection, neither IC-ELISA nor culture detected the influenza B in these samples. Among the 93 positive specimens, 40 were positive by IC-ELISA, 77 by tissue cell culture, 88 by PCR1, and 76 by PCR2 (Table I).

Among A H3N2 isolates, 84 were detected by PCR1 compared to 74 by PCR2. The 10 discrepant specimens were all negative by ELISA and four were also culture negative.

Five IC-ELISA positive samples were negative by culture; four of these (A H3N2), however, were positive in both PCR assays. The remaining sample (B) was negative by PCR1 (Table I).

All 76 PCR2 positive samples were positive using PCR1. However, 12 PCR1 positive samples were negative by PCR2 (9 influenza A H3N2 and 3 influenza B; Table I).

Using our conventional approach, which combines IC-ELISA plus culture, 82 samples were positive (53.6%). When compared with results obtained from PCR1, discrepancies were observed in 16 cases (10.4%): 5 (3.3%) were IC-ELISA/culture positive only (4 A H3N2 and 1 B), while 11 (7.1%) were PCR positive only (11 A H3N2). Statistical analysis of these discrepancies revealed that neither the population of patients screened/sampled (age, delay between onset of the disease and sampling, vaccination status, and epidemic cluster) nor differences in delay between the sampling and the processing of the swab could predict a specific group of patients or batch of swab specimens likely to be positive using one technique or the other.

In addition to influenza detection, the virological examination of samples revealed six RSV (including three mixed infection with A H3N2) five adenovirus (three type 3 and two type 7), one rhinovirus, one *Mycoplasma pneumoniae,* and one parainfluenza 3 infections. In total, virological data were obtained in 104/154 specimens (67.5%).

Results of the sensitivity of each test are shown in Table II. Sensitivities were adjusted to the volume of the suspension (or extraction) used for the detection; these volumes were 0.025 ml, 0.1 ml, and 0.3 ml for PCR, IC-ELISA, and culture, respectively. PCR 1 was more sensitive than PCR2 for H1N1 detection, while both PCRs were at least one log more sensitive than culture.

DISCUSSION

During the peak of the influenza epidemic, the rate of positive samples was very high, reaching up to 60% in a given week (Fig. 1). This result was obtained with the use of our conventional diagnostic scheme that combines antigenic detection (IC-ELISA) and cell culture. Influenza detection by PCR is useful for the detection of non-replicating viruses and in specimens with a low viral inoculum [Freymuth et al., 1997; Fan et al., 1998]. This was confirmed by the comparison of the four techniques carried out from calibrated viral suspensions.

In this study, the viruses we detected were A H3N2 Sydney-like strains, A H1N1 Beijing-like strains, and B Harbin-like strains. These are known to grow well in culture. The collection of the samples was performed by

TABLE II. Comparison of Sensitivities of Four Tests on Calibrated Viral Suspensions*

	Techniques			
Influenza type	IC-ELISA	Culture	PCR1	PCR2
A H1N1/Beijing 262/95	3,300	30	0.2	2
A H3N2/Sydney 5/97	5,000	45	3	3
B/Harbin 7/94	5,000	45	3	

*Values are in $TICD_{50}/\mu l$. Results were adjusted to the volume required for each test. IC-ELISA, immunocapture-ELISA assay; culture was done on MDCK cells and checked at day 4; PCR1 and PCR2 procedures are described in the text.

well-trained practitioners during the early stage of the disease. It is likely that most of the samples had a high influenza virus inoculum.

The most striking difference between PCR and culture techniques is the time-delay from setting up the test to having the results (24 hours for PCR, 4 days for culture). However, it is noteworthy that PCR detection did not significantly increase the number of positives. This could be due to the presence of high viral load in such GROG samples but may also reflect the presence of PCR inhibitors, proteases, or RNases that will alter RNA detection. The fact that PCR was undertaken on frozen specimens which had been stored at −20°C for 6 weeks may have reduced the detection rates.

With the development of antiviral therapy, clinical laboratories will need to use rapid diagnostic methods. As influenza culture requires four days, it is unsuitable for such a purpose. Using well-designed and comprehensive protocols, influenza detection by PCR is now available for any properly-trained laboratory. Small PCR machines are available, that can be used on a daily basis. Nested RT-PCR results can be obtained in 24 hours. Therefore, molecular diagnosis tests that allow accurate and sensitive detection of influenza are already implemented in numerous laboratories, PCR becoming an alternative to culture.

Nevertheless, even if our results confirm that PCR is a much more sensitive screening method than IC-ELISA, the optimised culture method that we employed showed an almost equivalent detection rate. This required good quality MDCK cells, careful checking and detection of influenza growth, and systematic antigenic detection of influenza in culture supernatants. Using this time consuming procedure (4 days), the rate of positive samples was almost comparable to this of PCR.

In large laboratories that process numerous specimens, detection by PCR remains an expensive option compared to antigenic detection or culture. For smaller laboratories, molecular detection is very attractive. Moreover, the development of multiplex PCR assays detecting different pathogens [Freymuth et al., 1997; Fan et al., 1998; Grondahl et al., 1998; Stockton et al., 1998] reduces the costs of detection per infectious agent. However, the problem involved in the use of multiplex PCR is the loss of sensitivity compared to running many individual pathogen-specific PCRs.

PCR detects but does not characterise influenza antigenic variants. For this constantly evolving and reemerging pathogen, such characterisation is important. Moreover, influenza strains that may undergo large antigenic drifts or antigenic shift will require a new set of optimised primers in order to be detected by PCR. This potential problem was highlighted by the detection of the first H5N1 strain from the 3-year-old boy in May 1997 in Hong-Kong. This A H5N1 strain was identified initially as influenza A by an immunofluorescence assay, and was not detected by H1 and H3 specific RT-PCR assays [Subbaro et al., 1998].

Therefore, optimised influenza surveillance requires sensitive detection of influenza strains and cultivation of isolates from infected patients. These objectives are not only those of reference laboratories but also those of diagnostic laboratories. The use of PCR is to have a sensitive test that assess rapidly the diagnosis of influenza in patients presenting with acute resperatory illness, but it must not prevent attempts to cultivate the virus; culture remaining the reference technique (the gold standard) for influenza diagnosis in laboratories.

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